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Mutation in GDP-Fucose Synthesis Genes of *Sinorhizobium fredii* Alters Nod Factors and Significantly Decreases Competitiveness to Nodulate Soybeans

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We mutagenized *Sinorhizobium fredii* HH103-1 with Tn*5*-B20 and screened about 2,000 colonies for increased β-galactosidase activity in the presence of the flavonoid naringenin. One mutant, designated SVQ287, produces lipochitooligosaccharide Nod factors (LCOs) that differ from those of the parental strain. The nonreducing *N*-acetylglucosamine residues of all of the LCOs of mutant SVQ287 lack fucose and 2-*O*-methylfucose substituents. In addition, SVQ287 synthesizes an LCO with an unusually long, C20:1 fatty acyl side chain. The transposon insertion of mutant SVQ287 lies within a 1.1-kb *Hin*dIII fragment. This and an adjacent 2.4-kb *Hin*dIII fragment were sequenced. The sequence contains the 3′ end of *noeK*, *nodZ*, and *noeL* (the gene interrupted by Tn*5*-B20), and the 5′ end of *nolK*, all in the same orientation. Although each of these genes has a similarly oriented counterpart on the symbiosis plasmid of the broad-host-range *Rhizobium* sp. strain NGR234, there are significant differences in the *noeK*/*nodZ* intergenic region. Based on amino acid sequence homology, *noeL* encodes GDP-D-mannose dehydratase, an enzyme involved in the synthesis of GDP-L-fucose, and *nolK* encodes a NAD-dependent nucleotide sugar epimerase/dehydrogenase. We show that expression of the *noeL* gene is under the control of NodD1 in *S. fredii* and is most probably mediated by the *nod* box that precedes *nodZ*. Transposon insertion into *noeL* has two impacts on symbiosis with Williams soybean: nodulation rate is reduced slightly and competitiveness for nodulation is decreased significantly. Mutant SVQ287 retains its ability to form nitrogen-fixing nodules on other legumes, but final nodule number is attenuated on *Cajanus cajan*.

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Nucleotide and/or amino acid sequence data are to be found at the EMBL data base as accession no. AF072888.

Sinorhizobium fredii was initially described as a fast-growing bacterium that nodulates soybean (*Glycine max* (L.) Merr.) in a cultivar-specific manner and that can enter into symbiosis with several other legume species (Keyser et al. 1982). Although the first, and many subsequently, isolated *S. fredii* strains were from China (Keyser et al. 1982; Dowdle and Bohlool 1985; Chen et al. 1988), similar microorganisms have been isolated from diverse geographical regions, including Panama (Hernandez and Focht 1984), Malaysia (Young et al. 1988), the United States (Shen and Davis 1992), and Vietnam (Rodriguez-Navarro et al. 1996). One of the most interesting characteristics of *S. fredii* is its exceptionally broad host range. Strain USDA257, for example, produces nitrogen-fixing nodules on more than 60 legume species (Krishnan and Pueppke 1994b; S. G. Pueppke, *unpublished*). *Rhizobium* sp. strain NGR234, a versatile symbiont that is closely related to and perhaps a strain of *S. fredii* (Jarvis et al. 1992), is even more promiscuous.

Although the unusual symbiotic properties of *S. fredii* make it an attractive model for experimental manipulation, genetic analysis of broad-host-range sinorhizobia is less advanced than that of narrower-host-range symbionts. Bacterial genes that function in nodulation are termed nodulation or *nod* genes, and their expression is induced by flavonoid signals from the plant host. The common *nodABC* and the regulatory *nodD1* and *nodD2* genes of *S. fredii* have been characterized (Appelbaum et al. 1988; Krishnan and Pueppke 1991; Krishnan et al. 1995). Nod factors, also known as lipochitooligosaccharides or LCOs, have also been isolated from *S. fredii*

and characterized (Bec-Ferté et al. 1994, 1996; Gil-Serrano et al. 1997). These signal molecules, which are synthesized by the protein products of the *nod* genes, regulate nodule initiation and morphogenesis (Dénarié et al. 1996).

There is evidence that the broad host range of *S. fredii* is fine-tuned by *nod* genes that function as determinants of host specificity. The *nodSU* locus, for example, is expressed in NGR234 but not in USDA257. This means that USDA257 does not synthesize Nod factors with *N*-methyl and *O*-carbamoyl substituents, which prevents nodulation of *Leucaena leucocephala*, a leguminous tree (Krishnan et al. 1992; Jabbouri et al. 1995). Other loci, such as *nolXWBTUV*, are probably not directly involved in Nod factor biosynthesis or secretion, yet they function negatively to regulate both cultivar specificity with soybean and nodulation of distantly related legumes (Heron et al. 1989; Meinhardt et al. 1993; Krishnan and Pueppke 1994a; Kovács et al. 1995; Bellato et al. 1997). Additional genes almost certainly are involved in defining other aspects of the host range of *S. fredii*, either by modifying Nod factor structure or by other mechanisms.

We report here the presence of two genes that are located on the symbiosis plasmid of *S. fredii* HH103 and resemble *nodZ* and *noeL* of *Rhizobium* sp. strain NGR234 (Freiberg et al. 1997). They are highly homologous to counterparts in strain NGR234, but the intergenic regions are divergent. The *nodZ* genes of other rhizobia encode fucosyltransferases (Stacey et al. 1994; López-Lara et al. 1996; Mergaert et al. 1996; Quesada-Vicens et al. 1997; Quinto et al. 1997), and the *noeL* gene is homologous to a GDP-D-mannose dehydratase gene of enteric bacteria. It thus seemed likely that these genes are involved in the biosynthesis of Nod factors in *S. fredii* and, indeed, we demonstrate that disruption of *noeL* leads to synthesis of nonfucosylated LCOs. Although this mutant retains the ability to form nitrogen-fixing nodules, its competitive capacity to nodulate soybean is decreased.

RESULTS

Isolation of Tn*5*-*lacZ* mutants with flavonoid-inducible β-galactosidase activity.

We employed suicide plasmid pSUP102-Gm carrying transposon Tn*5*-B20 (Simon et al. 1989) to randomly mutagenize *S. fredii* HH103-1. Two thousand Nm^r transconjugants were patched onto Bergersen's medium and assayed individually for elevated β-galactosidase activity in the presence of the flavonoid naringenin. Three presumptive mutants expressed higher β-galactosidase activity under these conditions, as assessed by the appearance of a dark blue color around the colonies. One of these clones, named SVQ287, was selected for detailed analysis. β-Galactosidase activity of mutant SVQ287 was about 120 Miller units (n = 4) in the absence of flavonoid inducers. When supplied with 3.7 μM naringenin, daidzein, and genistein, enzyme activity was elevated to 512, 525, and 640 U, respectively: a four- to fivefold level of induction.

Characterization of the Nod factors produced by HH103-1 and mutant SVQ287.

We used thin-layer chromatography (TLC) to analyze Nod factor preparations from cultures that had been induced with genistein in the presence of ^{14}C-glucosamine. At least five spots were resolved from extracts of parental strain HH103-1, but only two were evident in extracts of SVQ287 (Fig. 1A). There are also differences in the migration of individual compounds. TLC analysis was also performed with (methyl-^{14}C)-L-methionine as radioactive label. The LCOs produced by strain HH103-1 became labeled under these conditions, but those of SVQ287 did not (Fig. 1B). Collectively, these observations suggest that the Nod factors produced by SVQ287 are quantitatively and qualitatively different from those of the parental strain and that they probably lack methyl substituents.

The Nod factors of mutant SVQ287 were purified by high-performance liquid chromatography (HPLC) into five fractions (designated I to V). Gas chromatography-mass spectrometry (GC-MS) of the trimethylsilyl ethers of methyl glycosides identified only a single hexosamine, which was confirmed to be D-glucosamine following GC-MS analysis of its trimethylsilylated (+)-2-butyl glycoside and (±)-2-butyl glycosides. Methylation analysis of the total LCO extract confirmed the presence of 1,5-di-*O*-acetyl-3,4,6-tri-*O*-methyl-*N*-acetyl-*N*-methylglucosaminitol from the nonreducing terminal glucosamine residue and 1,4,5-tri-*O*-acetyl-3,6-di-*O*-methyl-*N*-acetyl-*N*-methylglucosaminitol from the internal and reducing terminal glucosamine residues.

The fatty acid analysis identified hexadecanoic acid (C16:0), octadecanoic acid (C18:0), hexadecenoic acid (C16:1), octadecenoic acid (C18:1), and icosenoic acid (C20:1). The mass spectrum of the dimethyldisulfide derivatives of the unsaturated fatty acid methyl esters (data not shown) contain for C18:1 ions at *m/z* 390 (molecular ion), and *m/z* 145 and 245, arising from the fragmentation between the carbons that carry the dimethyldisulfide groups, and demonstrate that the double bond is at carbon 11. For C16:1 the analogous ions are at *m/z* 362, 145, and 217. The presence of the fragment ions at *m/z* 145 and 217 demonstrates that the double bond is located at carbon 9. The position of the double

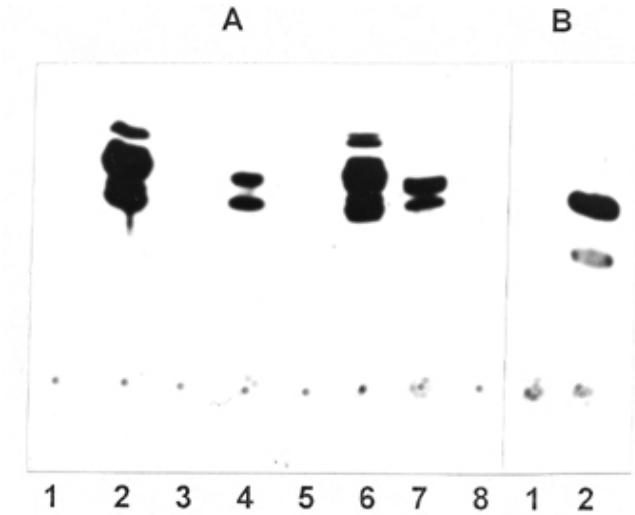


Fig. 1. TLC (thin-layer chromatography) analysis of the lipochitooligosaccharides (LCOs) produced by *Sinorhizobium fredii* HH103-1, its mutant derivative SVQ287, and strain SVQ287(pMUS283). **A,** ^{14}C-glucosamine as label in (lanes 1, 3, 5, and 8) the absence or (lanes 2, 4, 6, and 7) presence of 3.7 μM genistein as inducer. Lanes 1 and 2, HH103-1; lanes 3 and 4, SVQ287; lanes 5 and 6, SVQ287(pMUS283); lanes 7 and 8, SVQ287(pMUS475). **B,** (Methyl-^{14}C)-L-methionine as label with 3.7 μM genistein as inducer. Lane 1, SVQ287; lane 2, HH103-1.

bond in C20:1 was not determined because of the small amount of the sample. Fraction FI contains C16:1, C18:1, and C18:0; FII contains C16:0, C18:0, and C18:1; FIII contains C16:0 and C18:1; FIV contains only C18:1; and FV contains C18:0, C18:1, and C20:1

The results of analysis of fractions I–V by fast atom bombardment (FAB)-MS are summarized in Table 1. $[M+H]^+$ and $[M+Na]^+$ pseudomolecular ions were present, as were the thioglycerol adduct ions $[M+H+TG]^+$ and $[M+Na+TG]^+$ in the case of LCOs with unsaturated fatty acids. The positive ion collision-induced dissociation tandem (CID-MS-MS) mass spectra contain oxonium-type fragment ions formed by sequential cleavage of each glycosidic linkage, with charge retention on the nonreducing portion of each ion. The *m/z* value of the lowest mass oxonium ion allows the identification of the fatty acyl group on the nonreducing terminal *N*-acetylglucosamine residue. We have identified a total of 10 LCOs synthesized by mutant SVQ287, all of which are devoid of fucose and 2-*O*-methylfucose (Fig. 2). Fucose- or 2-*O*-methylfucose-containing homologues of eight of these structures are elaborated by the parental strain HH103-1 (Gil-Serrano et al. 1997), but homologues of the other two were not identified. The compounds are Sf-IV(C20:1) and Sf-VI(C18:1). Conversely, nonfucosylated counterparts of three LCOs of strain HH103 were not identified from the mutant.

Symbiotic phenotype of mutant SVQ287.

The symbiotic properties of mutant SVQ287 were assessed with six legumes known to undergo effective nitrogen-fixing symbioses with the *S. fredii* wild-type strain HH103. Mutant SVQ287 retains the ability to produce nitrogen-fixing nodules on each of these plants, but the number of nodules per plant on *Cajanus cajan* is obviously reduced (Table 2). The mutant was also tested with five legume species not nodulated by strain HH103 (*Albizia lophantha, Galega orientalis, Lablab purpureus, Leucaena leucocephala,* and *Vigna aconitifolia*). None of these plants was nodulated.

Mutant SVQ287 forms nitrogen-fixing nodules with the soybean cultivar Williams; nitrogenase activity at 6 weeks after inoculation was only 82% of that of parental strain HH103-1, but this difference is not statistically significant. The corresponding shoot dry weight of plants inoculated with SVQ287 was 72% of that of plants inoculated with HH103-1, but the decrease again is not significant. The initial nodulation rate of plants inoculated with SVQ287 was slightly retarded, but the ultimate number of nodules produced by SVQ287 was not different from that produced by its parental strain HH103-1. This is reflected by the mean number of nodules per plant for HH103-1 versus SVQ287: 2.2 versus 1.8 at day 9, 12.2 versus 7.2 at day 14, 35.0 versus 31.8 at day 19, 38.6 versus 32.6 at day 23, and 53.0 versus 57.4 at day 30.

The difference in nodulation rates prompted us to compare the capacities of the mutant and parent to nodulate Williams soybean in competition with one another. Mutant SVQ287 was outcompeted by parental strain HH103-1 at three different inoculum ratios (Table 3). Even when SVQ287 was supplied in 10-fold numerical excess, 73% of the nodules contained only the parental strain, indicating that the mutation influences the ability of the bacterium to interact with roots. Reisolates that were Str^rKm^r (presumed SVQ287) and Str^rKm^s (presumed HH103-1) were subjected to TLC analysis for production of LCOs in the presence of genistein. All retained the expected patterns (data not shown), indicating that the mutated gene region of SVQ287 had not been altered during the course of the experiment.

Genetic and sequence analysis of the mutated region in strain SVQ287.

In a separate study, we isolated a deletion mutant of *S. fredii* USDA192 that lacks *nodD1* and an unknown number of linked genes. This mutant does not produce Nod factors and does not nodulate. We used *nodD1*-containing cosmid pMUS283 from strain HH103 to complement the mutation for nodulation and found that the transconjugant containing cosmid pMUS283 synthesizes a normal profile of Nod factors (as determined by TLC analysis), one virtually identical to that of strains HH103 and USDA192. However, the same nonnodulating deletion mutant carrying a subfragment of cosmid pMUS283 that contains only *nodD1* produces a set of Nod factors that is very similar (in TLC experiments) to that produced by mutant strain SVQ287. The fortuitous similarity between the two sets of Nod factors suggested that the gene disrupted in SVQ287 might be linked to *nodD1*, and so we transferred pMUS283 to the mutant. TLC analysis with ^{14}C-

Table 1. FAB-MS (fast atom bombardment-mass spectrometry) and collision-induced dissociation tandem (CID-MS-MS) analysis of lipochitooligosaccharides (LCOs) from *Sinorhizobium fredii* SVQ287

Fraction	[M+H][a]	[M+Na][a]	[M+H+TG][a]	[M+Na+TG][a]	Oxonium-type ions	Designation
I	1053	1075	1161	1183		Sf-IV(C18:1)
	1055M	1077				Sf-IV(C18:0)
	1228	1250	1336	1358		Sf-V(C16:1)
II	1053		1161			Sf-IV(C18:1)
	1055					Sf-IV(C18:0)
	1230	1252				Sf-V(C16:0)
	1256	1278	1364	1386		Sf-V(C18:1)
	1459	1481	1567	1589		Sf-VI(C18:1)
III	1027	1049			400, 603, 806	Sf-IV(C16:0)
	1256	1278	1364	1386	426, 629, 832, 1035	Sf-V(C18:1)
IV	1053	1075	1161	1183	426, 629, 832	Sf-IV(C18:1)
V	850	872	958	980		Sf-III(C18:1)
	1055M	1077			428, 631, 834	Sf-IV(C18:0)
	1081m		1189			Sf-IV(C20:1)
	1258	1280				Sf-V(C18:0)

[a] M, Major component in each fraction; m, minor component in each fraction.

glucosamine as radioactive label confirmed that the LCOs of strain SVQ287(pMUS283) were indistinguishable from those of parental strain HH103-1 (Fig. 1A).

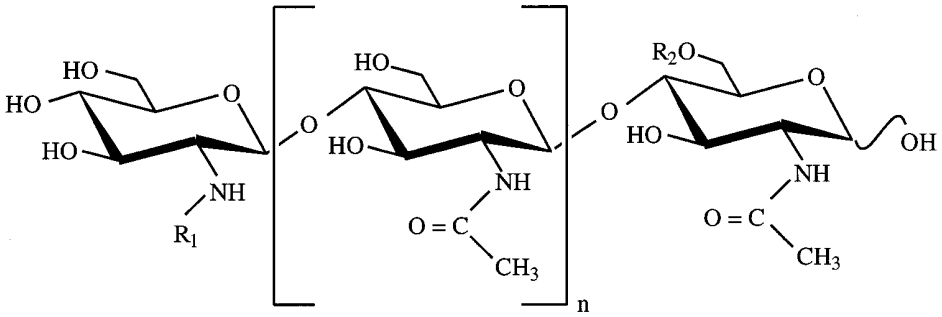
A 9.6-kb *Eco*RI fragment from pMUS283 (Fig. 3A) was subcloned into plasmid pMP92 to generate plasmid pMUS424, which was subsequently conjugated into mutant SVQ287. ^{14}C-labeled LCOs then were resolved by TLC. The pattern of genistein-induced LCOs was indistinguishable from that produced by SVQ287(pMUS283) or HH103-1 (Fig. 1A). A 2.7-kb *Apa*I fragment that lies within the 9.6-kb *Eco*RI fragment (Fig. 3A) was subcloned into plasmid pMP92 to generate pMUS475. In contrast to pMUS424, this plasmid did not complement the mutation of SVQ287 as assessed by LCO production (Fig. 1A).

A genomic library, constructed in the cosmid vector pLAFR1, was employed to localize the transposon insertion in strain SVQ287. The library was plated onto LB medium supplemented with tetracycline and kanamycin, to select the cosmid vector and transposon, respectively. Four Tc^r Km^r *Escherichia coli* clones were randomly selected and their cosmids purified and digested with *Eco*RI. Agarose gel electrophoresis demonstrated that each cosmid contained a common hybridizing *Eco*RI fragment of 11 kb, which conferred Km^r upon subcloning and transfer back into *E. coli*. One of these cosmids was named pMUS519, and the exact position of transposon Tn*5-lacZ* in it was determined by sequencing outward from the terminus of IS50R and into the adjacent DNA from *S. fredii*. Simultaneously, we began sequencing wild-type plasmid pMUS424 (Fig. 4A) and, by matching se-

Table 2. Symbiotic responses to inoculation with *Sinorhizobium fredii* HH103-1 and its mutant derivative SVQ287[a]

Legume specis	Phenotype		No. plants
	HH103-1	SVQ287	
Albizia lebbeck	Fix$^+$	Fix$^+$	4
Cajanus cajan[b]	Fix$^+$	Reduced nodulation	8
Desmodium canadense	Fix$^+$	Fix$^+$	4
Glycine max cv. Williams	Fix$^+$	Fix$^+$	10
Macroptilium atropurpureum	Fix$^+$	Fix$^+$	10
Neonotonia wightii	Fix$^+$	Fix$^+$	14

[a] Determinations were made 5 to 7 weeks after inoculation.
[b] HH103-1 and SVQ287 formed an average of 80 and 12 nitrogen-fixing nodules per plant, respectively.



S. fredii SVQ287 *S. fredii* HH103

n	R_1 (fatty acid)	R_2	n	R_1 (fatty acid)	R_2
	-		1	C16:1Δ9	2-*O*-Me-Fuc
	-		2	C16:1Δ9	Fuc and 2-*O*-Me-Fuc
3	C16:1Δ9	H	3	C16:1Δ9	Fuc and 2-*O*-Me-Fuc
2	C16:0	H	2	C16:0	2-*O*-Me-Fuc
3	C16:0	H	3	C16:0	Fuc and 2-*O*-Me-Fuc
1	C18:1Δ11	H	1	C18:1Δ11	2-*O*-Me-Fuc
2	C18:1Δ11	H	2	C18:1Δ11	Fuc and 2-*O*-Me-Fuc
3	C18:1Δ11	H	3	C18:1Δ11	Fuc and 2-*O*-Me-Fuc
4	C18:1Δ11	H		-	-
			1	C18:0	2-*O*-Me-Fuc
2	C18:0	H	2	C18:0	2-*O*-Me-Fuc
3	C18:0	H	3	C18:0	2-*O*-Me-Fuc
2	C20:1	H		-	-

Fig. 2. Summary of the lipochitooligosaccharide (LCO) structures produced by mutant derivative SVQ287 compared with those from strain HH103 (Gil-Serrano et al. 1997).

quences, were able to position the transposon at nucleotide 2,956 of the final 3,506 nucleotide sequence. This sequence covers two *Hin*dIII fragments, as shown in Figure 4, and appears in the EMBL nucleotide sequence data library as accession no. AF072888.

The fragment sequenced contains two adjacent open reading frames (ORFs), both of the same polarity and with a high probability of encoding proteins, as indicated by the Testcode algorithm. These two ORFs are highly homologous to *nodZ* and *noeL* of *Rhizobium* sp. strain NGR234 (Freiberg et al. 1997). The right and left ends of the fragment also contain homologues of the 3′ terminus of *noeK* and the 5′ terminus of *nolK* of strain NGR234, respectively (Fig. 3B).

nodZ begins at position 912 and extends to the right for 966 bp, encoding a deduced polypeptide of 322 amino acids with a predicted size of 36.5 kDa. This gene bears a striking similarity to the corresponding gene of *Rhizobium* sp. strain NGR234 (Freiberg et al. 1997), having only four mismatches in the primary nucleic acid sequence and three in the deduced amino acid sequence of the protein (Fig. 5). As in NGR234, the initiation codon for NodZ of HH103 appears to be TTG. The ORF is preceded by a putative ribosome binding site, AAGGACGG, at positions –14/–7 (Fig. 4). NodZ of *Azorhizobium caulinodans* is nearly the same length as the *S. fredii* protein (Mergaert et al. 1996), but the corresponding protein of *Bradyrhizobium japonicum* (López-Lara et al. 1996) is 47 amino acids longer (Fig. 5). The amino acid identity between NodZ of *S. fredii* and the homologous proteins from *A. caulinodans* and *B. japonicum* is 45.2 and 70.1%, respectively.

noeL begins 245 bp downstream from *nodZ* at position 2,125 and extends for 1,053 bp to the TAA stop codon at position 3,178. Mutant SVQ287 contains the transposon insertion at the 5′ end of the *noeL* gene (at position 2,956). It encodes a putative protein of 351 amino acids with a predicted size of 40.0 kDa. Only 11 nucleotides and four amino acid substitutions differentiate *noeL* of *S. fredii* from *noeL* of NGR234 (Freiberg et al. 1997). The GTG initiation codon is preceded by the putative ribosome binding site, GGAGAT, at positions –14/–9. Computer analysis points to substantial homology between NoeL of *S. fredii* and GDP-D-mannose dehydratase genes of several enteric bacteria (Fig. 6), as well as other organisms (data not shown). The NoeL proteins of strains HH103 and NGR234 nevertheless lack an internal fragment of 10 amino acids that characterizes the enteric proteins (Fig. 6).

Table 3. Competition between *Sinorhizobium fredii* mutant SVQ287 and parental strain HH103-1 for nodulation of Williams soybean[a]

HH103-1:SVQ287 (ratio in inoculum)	HH103[b]	SVQ287[b]	Both[b]	SVQ287[c]
10:1	94	1	5	10
1:1	95	3	2	50
1:10	73	18	9	90

[a] Ten plants were inoculated with each strain combination, and nodule occupancy was determined 5 weeks later. Strain HH103-1 and mutant SVQ287 were identified as Km^s and Km^r colonies, respectively. The value of 1 in each ratio corresponds to 10^8 bacteria per ml.

[b] Percentage of nodules containing strain. A total of 118, 105, and 100 nodules, respectively, were examined for each of the three treatments.

[c] Percentage expected to contain this strain, calculated on the basis of the initial inoculum ratio.

nodZ and *noeL* are flanked by two other partially sequenced genes that have homologues in NGR234. The left side of the sequenced *Hin*dIII fragment contains 594 bp from the 3′ end of an ORF that is 99.0% identical to *noeK* (Fig. 3B). The last two nucleotides of the TAA stop codon of *noeL* overlap a putative ribosome binding site, AAGAGG, that precedes an additional ORF, only part of which was sequenced. This 318-bp partial ORF is 99.0% identical to the *nolK* gene of *Rhizobium* sp. strain NGR234.

The intergenic region between *noeK* and *nodZ* is 317 bp long in *S. fredii* HH103 and represents the only portion of the sequenced region that differs substantially from the corresponding region of NGR234 (Fig. 4). The first 55 bp of the intergenic region are identical in both rhizobia, but this is followed by a 74-bp block that is absent in NGR234. The remaining 188 bp, however, contain only four mismatches. The unique 74-bp segment contains a possible ORF that encodes an oligopeptide of 22 amino acids. This polypeptide has homology (50% identity and 55% charge similarity) to the first 20 amino acids of the NifW protein of *Klebsiella pneumoniae,* an enteric bacterium that fixes nitrogen in the free-living state (Arnold et al. 1988). There is no significant homology to NifW of strain NGR234 (Freiberg et al. 1997).

Expression of *noeL* is *nodD1*-dependent.

nodZ of *B. japonicum* is under the control of a promoter that does not contain a *nod* box, and as a result, expression is independent of the NodD regulatory circuit (Stacey et al. 1994). In contrast, the *nodZ*/*noeL* loci of *S. fredii* HH103 and *Rhizobium* sp. strain NGR234 both are preceded by a highly conserved *nod* box (Spaink et al. 1987; Goethals et al. 1992) and an *nolR* box, which also may be involved in regulation (Kondorosi et al. 1991). This makes it conceivable that *nodZ* and *noeL* are under the control of NodD in *S. fredii*. This was tested by transferring cosmid pMUS519 into strain SVQ318, a *nodD1* mutant of HH103, and into strain HH103-1. As expected, β-galactosidase activity of HH103-1 (pMUS519) was higher in the presence of genistein (3,969 Miller units) than in the absence of inducer (589 Miller units). The β-galactosidase activity of strain SVQ318(pMUS519), in contrast, was nearly the same in the presence of genistein (445 Miller units) as in its absence (465 Miller units).

DISCUSSION

We have employed transposon Tn*5-lacZ* mutagenesis to identify genes with elevated expression in response to flavonoid signal molecules from the plant host. Although this strategy to identify genes of potential symbiotic significance is laborious, it has the advantage of randomness. This is an important consideration for *S. fredii*, a broad-host-range organism from which very few host specificity genes have been isolated. Our secondary screen, TLC analysis of LCOs, facilitated identification of genes involved in the synthesis of bacterial Nod factors. Ultimately, assessment of symbiotic phenotypes with a range of legumes made it possible to link structural alterations in LCOs to nodulation. Following this general strategy, we have screened 2,000 *S. fredii* mutants, each carrying a random Tn*5-lacZ* insertion. We confined our initial analysis to one mutant, SVQ287, that shows an increase in β-galactosidase activity in the presence of the inducers genistein, daidzein, and naringenin.

The most striking feature of the LCOs of mutant SVQ287 is the absence of fucosyl or 2-*O*-methylfucosyl residues. Synthesis of GDP-L-fucose from GDP-D-mannose is a three-step process in *E. coli* (Gabriel 1973). First, GDP-D-mannose dehydratase converts GDP-D-mannose into GDP-4-keto-6-deoxy-D-mannose. The final two steps in the pathway are the epimerization of GDP-4-keto-6-deoxymannose at C-3 and C-5 and then reduction at C-4. Mutation of *nolK* or *nodZ* abolishes fucosylation of LCOs (López-Lara et al. 1996; Mergaert et al. 1996; Quesada-Vincens et al. 1997), and there is evidence that NolK is involved in conversion of GDP-4-keto-6-deoxy-D-mannose into GDP-L-fucose (Mergaert et al. 1996). NodZ, in

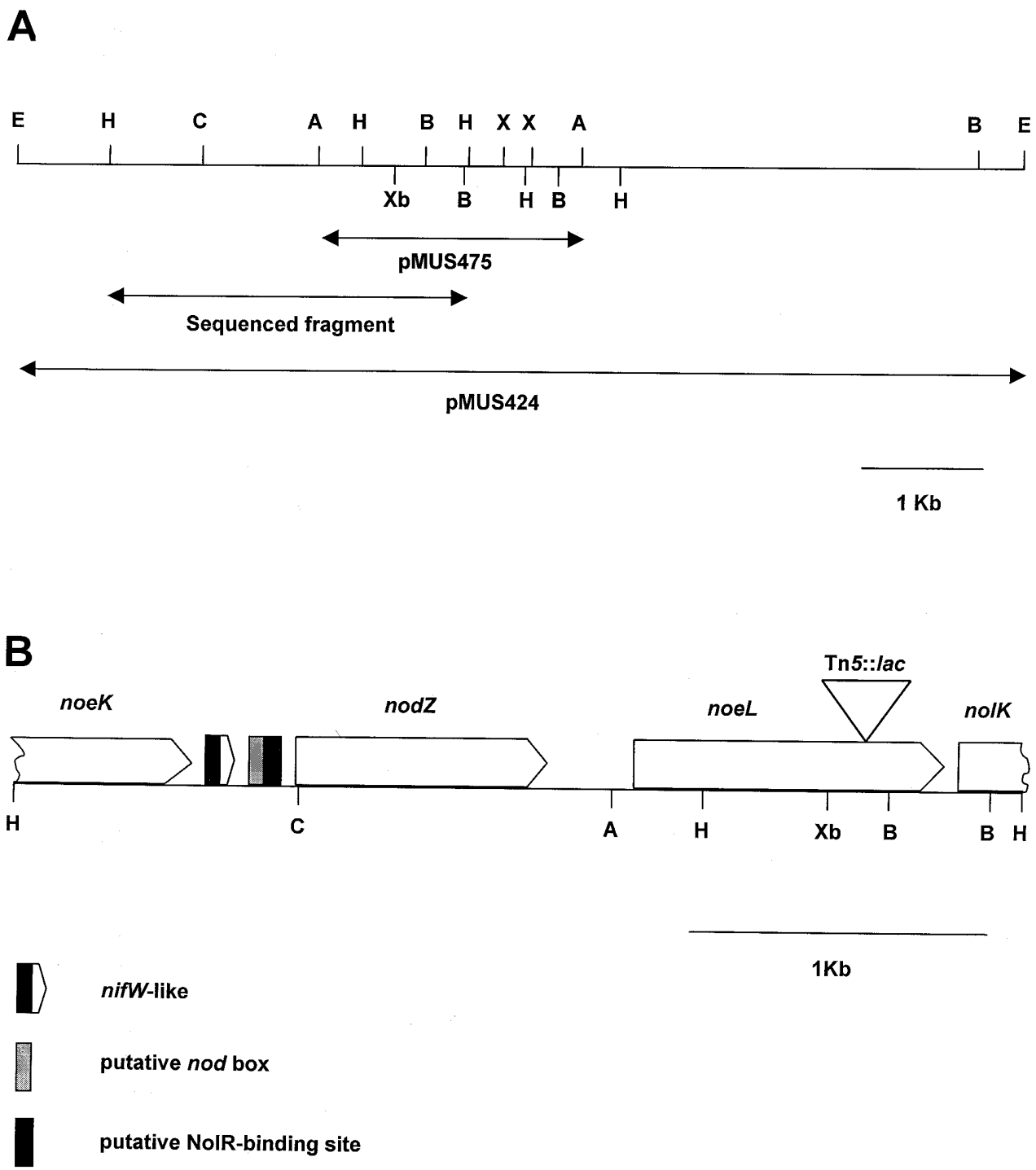


Fig. 3. A, Restriction map of the 9.6-kb *Eco*RI fragment of cosmid pMUS283 that contains the *nod* genes responsible for fucosylation of lipochitooligosaccharides (LCOs) by *Sinorhizobium fredii* HH103. **B,** Orientation of open reading frames (ORFs) and other features on the 3.5-kb *Hin*dIII fragment sequenced. Abbreviations for restriction enzymes: A = *Apa*I, B = *Bam*HI, C = *Cla*I, E = *Eco*RI, H = *Hin*dIII, X = *Xho*I, Xb = *Xba*I.

contrast, appears to be a fucosyl transferase (López-Lara et al. 1996; Mergaert et al. 1996; Quesada-Vincens et al. 1997; Quinto et al. 1997).

Mutant SVQ287 carries a Tn*5-lacZ* insertion in *noeL* (Freiberg et al. 1997), the protein product of which is homologous to GDP-D-mannose dehydratase. Inactivation of *noeL* thus would be expected to prevent the addition of the fucosyl residue to bacterial LCOs, provided that no other copies of the gene are present elsewhere in the genome. It is nevertheless possible that the insertion has polar effects on downstream genes such as *nolK* and that this influences Nod factor structure.

gmd, the *E. coli* homologue of *noeL*, functions in the synthesis of colanic acid, an extracellular polysaccharide that is produced by most *E. coli* strains and other species of the family Enterobacteriaceae. The colony morphology of SVQ287 is not visibly different from that of its parental strain, and lipopolysaccharides produced by SVQ287 and HH103 are indistinguishable as assessed by polyacrylamide gel electrophoresis (data not shown). Disruption of *noeL* thus has no apparent gross effect on the bacterial surface, and so symbiotic alterations associated with the mutation are more likely to be due to changes in the LCOs than to pleiotropic effects on the cell surface. Quesada-Vincens and associates (1997) also have evidence that the symbiotic phenotype of *nodZ* mutants of NGR234 is directly attributable to alterations in LCO structure.

A total of 10 and 16 LCO structures have been identified from mutant SVQ287 and parent strain HH103, respectively, with structural variation not being limited to the simple presence or absence of fucosyl substituents. Both organisms produce sets of LCOs (Fig. 2) that differ in their fatty acyl substituent (stearic acid, vaccenic acid, palmitoleic acid, or palmitic acid) and the length of the oligochitin backbone of the molecule (tri-, tetra-, or pentameric). Only one of the 12 possible combinations of fatty acid and oligochitin chain, the C16:0 trimer, SfNod-III(C16:0, fuc) was not identified from strain HH103. In contrast, four of these basic structures were not identified from the mutant. However, LCO molecules not identified from HH103 were detected in the mutant strain. One of them, NodSf-VI(C18:1), is a member of the vaccenic acid family but has an unusually long hexameric oligochitin backbone. The second structure, NodSf-IV(C20:1), is a tetramer with an atypical 20 carbon fatty acyl substituent. Neither of these molecules has been identified previously as a metabolite of *Sinorhizobium* spp. (Roche et al. 1991; Price et al. 1992; Schultze et al. 1992; Bec-Ferté et al. 1994, 1996; Lorquin et al. 1997a, 1997b).

The expression of *noeL* is enhanced by flavonoids in mutant SVQ287 but not in a *nodD1*-negative background, indicating that this gene is NodD-dependent. Transcription of the *noeL* gene is likely to be controlled by the *nod* box that precedes *nodZ* (Fig. 3B), as has been proposed for the homologous gene from strain NGR234 (Fellay et al. 1995). This conclusion is supported by the fact that plasmid pMUS475, which contains the complete *noeL* coding region, 96 bp of the *nodZ-noeL* intergenic region, but no *nod* box, fails to complement the mutation of SVQ287.

There is remarkable homology between the ORFs sequenced from *S. fredii* HH103, a Chinese strain, and those of *Rhizobium* sp. strain NGR234, a New Guinea strain that is not able to form nitrogen-fixing nodules with soybean. Such similarity between the symbiosis plasmids of HH103 and NGR234 is not just restricted to the genes involved in the fucosylation of LCOs. Other genes sequenced from *S. fredii* HH103 and USDA257 show very high homology with counterparts of strain NGR234, e.g., *nodD1* and *nodD2* (Krishnan et al. 1995), *nodABC* (Krishnan and Pueppke 1991),

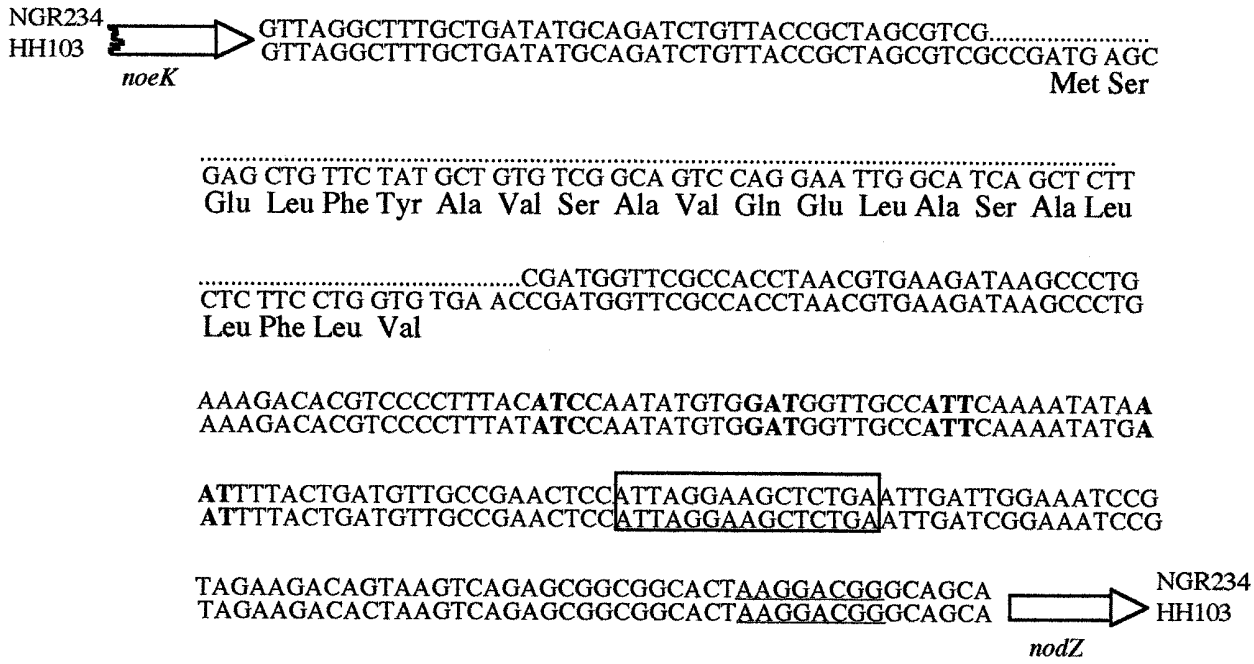


Fig. 4. Nucleotide and deduced amino acid sequences deduced of the *nifW*-like open reading frame (ORF) that lies within the *noeK-nodZ* intergenic region. The putative NolR-binding site is boxed; the putative ribosome-binding site of nodZ is underlined. The characteristic ATC, GAT, ATT, and AAT nucleotide blocks within the *nod* box (Goethals et al. 1992) are in boldface.

nolXWBTUV (Meinhardt et al. 1993), and the ORFs designated y4iR and y4vC (R. A. Bellogín, R. Espuny, F. J. Ollero, and J. E. Ruiz-Sainz, *unpublished results*). The fact that even the third base of the codons is so well preserved suggests that horizontal gene transfer between these bacteria has occurred recently. Mergaert and associates (1996) have drawn similar conclusions about the acquisition of *nod* genes by *Azorhizobium caulinodans*. It is nevertheless possible to detect significant variations between the *noeK-nodZ* intergenic regions of *S. fredii* HH103 and *Rhizobium* sp. strain NGR234. Strain HH103 contains an extra DNA fragment that it is not present in NGR234, and this fragment shows homology to the first 60 bp of *nifW* of *Klebsiella pneumoniae* but not the corresponding gene from NGR234. We have found similar insertions in the intergenic region between the ORFs designated y4vC and vf1 (Freiberg et al. 1997; R. A. Bellogín, R. Espuny, F. J. Ollero, and J. E. Ruiz-Sainz, *unpublished results*).

Mutant SVQ287 forms nitrogen-fixing nodules on six legumes that are nodulated by the parental strain. The competitive capacity of the mutant to nodulate soybean, the host from which strain HH103 was isolated (Dowdle and Bohlool 1985),

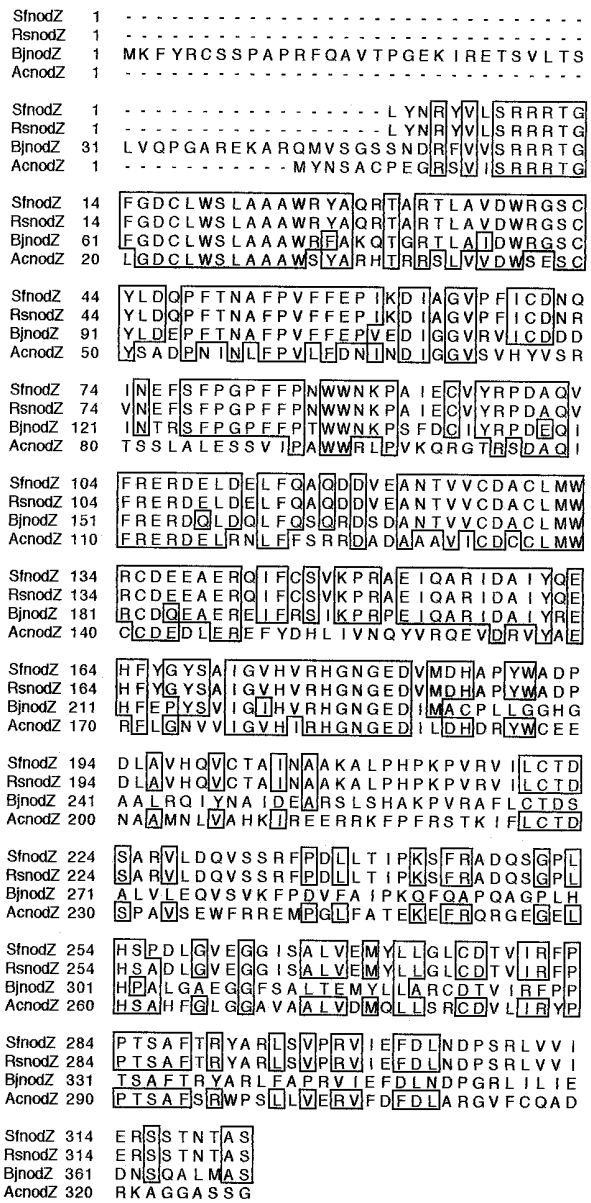


Fig. 5. Alignment of the NodZ proteins of *Sinorhizobium fredii* HH103 (SfnodZ), *Rhizobium* sp. strain NGR234 (RsnodZ), *Bradyrhizobium japonicum* (BjnodZ), and *Azorhizobium caulinodans* (AcnodZ). Conserved amino acids are boxed.

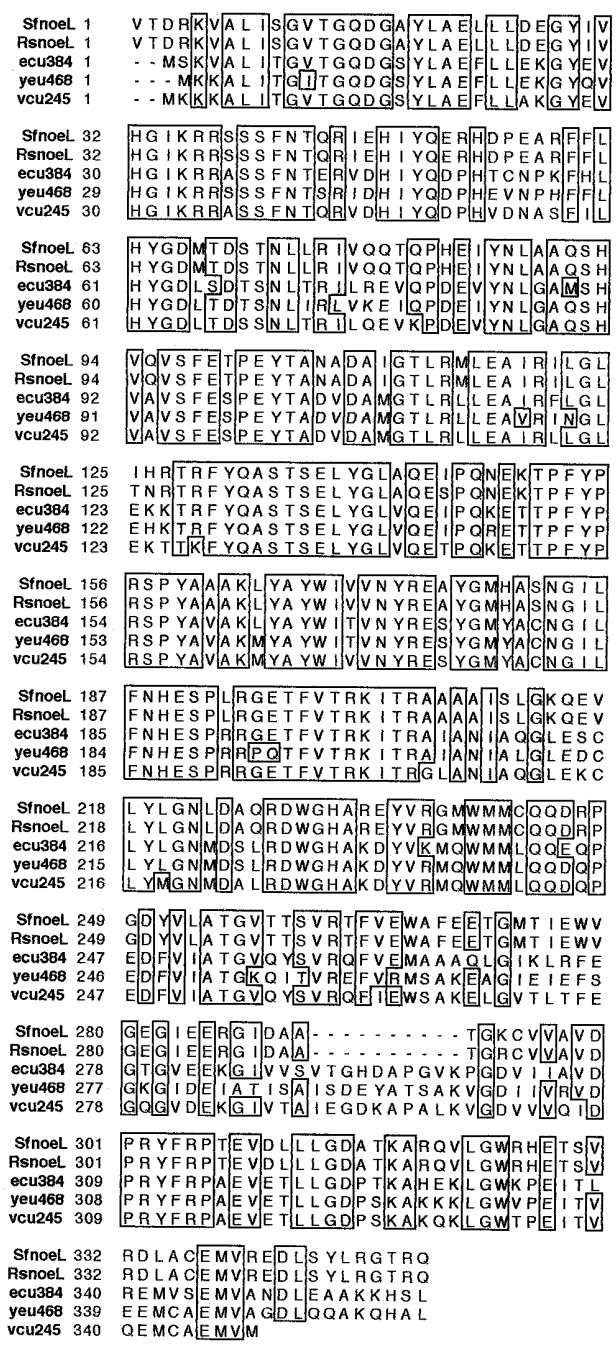


Fig. 6. Alignment of NoeL of *Sinorhizobium fredii* (SfnoeL) and *Rhizobium* sp. strain NGR234 (RsnoeL) with GDP-D-mannose dehydratase of *Escherichia coli* (ecu384), *Yersinia enterocolitica* (yeu468), and *Vibrio cholerae* (vcu245). Conserved amino acid residues are boxed.

is greatly compromised. Symbiotic capacity for *Cajanus cajan* is also reduced, as measured by final nodule numbers. Although *C. cajan* forms indeterminate nodules (Allen and Allen 1981), nodulation of *Albizia lebbeck*, another indeterminate nodule-forming species, is unaffected. It thus seems clear that the ability of HH103 to nodulate does not depend on the production of fucosylated LCOs, but that these molecules may play important ancillary roles in nodulation efficiency. Although fucosylation of Nod factors appears to play a similar accessory role in nodulation by *B. japonicum* (Stacey et al. 1994), a *nodZ*-negative mutant of strain NGR234 apparently fails to nodulate *Pachyrhizus tuberosus* (Quesada-Vincens et al. 1997).

MATERIALS AND METHODS

Strains, plasmids, and media.

The bacterial strains and plasmids used in this study are listed in Table 4. *Sinorhizobium* strains were cultured in TY medium (Beringer 1974) or minimal medium (Bergersen 1961). *E. coli* strains were grown in Luria-Bertani (LB) medium (Maniatis et al. 1982). When required, the media were supplemented with antibiotics (amounts in μg ml^{-1}): rifampicin, 50; streptomycin, 400; kanamycin, 50 (25 for *E. coli*); neomycin, 100; tetracycline, 4 (10 for *E. coli*); nalidixic acid, 20.

Genetic techniques.

Plasmids were transferred by conjugation as described by Simon (1984). To carry out random transposon Tn*5*-*lacZ* mutagenesis, plasmid pSUP102-Gm (carrying the Tn*5*-*lacZ* transposon Tn5-B20) was transferred by membrane crosses from *E. coli* S17-1 to strain HH103-1. Nm^r transconjugants were screened for β-galactosidase activity that could be induced by 3.7 μM naringenin or genistein. This was assessed by the appearance of a dark blue color surrounding bacterial patches. Assays for β-galactosidase activity in liquid bacterial cultures were as described by Spaink et al. (1987).

Total genomic DNA and large- and mini-scale plasmid and cosmid DNA preparations were as described by Maniatis et al. (1982). DNA manipulation, including restriction digestion, ligation, transformation, electrophoresis, and bacterial transformation were performed according to the general protocols of Maniatis et al. (1982). A cosmid library of mutant SVQ287 in pLAFR1 was constructed as described (Heron et al. 1989) and maintained in *E. coli* HB101.

Nod factor (LCO) purification.

Ten liters of culture medium was extracted with 3 liters of n-butanol. The butanol extract was evaporated to dryness under vacuum and the residue was suspended in 50 ml of 3:2 acetonitrile-water that was then brought to 1:4 acetonitrile-water. A prepurification step was performed by passing the crude extract through a Super Clean LC18, Supelco column (Supelco, Bellefonte, PA) from which the LCOs were eluted with different acetonitrile-water ratios (20, 45, and 60%). These fractions were purified by HPLC on a semi-preparative C18 reversed phase column (250 × 7.5 mm, Spherisorb ODS2, 5 mm, Tracer, Barcelona, Spain) with isocratic elutions of acetonitrile-water (20%, 5 min; 30%, 30 min; 40%, 30 min; 60%, 15 min), and then a linear gradient over 10 min from 60 to 100% of acetonitrile. The eluent from the HPLC was monitored at 206 nm.

Carbohydrate composition and methylation analysis.

Glycosyl composition analysis was carried out after methanolysis with anhydrous methanolic 0.625 M HCl (16 h, 80°C). The samples were re-acetylated with 1:1 (vol/vol) acetic anhydride-pyridine then trimethylsilylated with 1:1 (vol/vol) pyridine-BSTFA and analyzed by GC-MS (Chaplin 1982). The absolute configuration of the glucosamine was assigned following GC-MS analysis of its trimethylsilylated 2-butylglycosides prepared with (+)-2-butanol and (±)-2-butanol (Gerwig et al. 1978) as above. The LCOs were permethylated (Ciucanu and Kerek 1984) and the samples hydrolyzed with 2 M trifluoroacetic acid (120°C, 1 h), reduced, and acetylated by the method of Blakeney et al. (1984). The permethylated alditol acetates were analyzed by GC-MS. The GC-MS instrument, the columns, and analytical conditions for the different derivatives have been described previously (Gil-Serrano et al. 1995).

Fatty acid analysis.

The fatty acids were identified as methyl esters. The methyl esters were prepared by methanolysis in methanolic 0.625 M

Table 4. Bacterial strains and plasmids

Strain	Derivation and relevant properties	Source or reference
Sinorhizobium fredii		
HH103	Wild type	Buendía-Clavería et al. 1994
HH103-1	HH103 Str^r	Buendía-Clavería et al. 1989
SVQ287	HH103-1 *noeL*::Tn*5*-B20	This work
SVQ318	HH103-1 *nodD1*	Vinardell 1997
Escherichia coli		
S17-1	294 Rec^-, chromosomally integrated RP4 derivative, Tp^r Sm^r	Simon et al. 1983
HB101	Restriction-minus; *recA* background	Boyer and Roulland-Dossoix 1969
Plasmids		
PSUP102-Gm::Tn*5*-B20	Suicide plasmid carrying the Tn*5*-*lacZ* transposon Tn*5*-B20	Simon et al. 1989
PMUS283	Cosmid pLAFR1 carrying nodulation genes of *S. fredii* HH103	This work
PMUS424	pMP92 containing a 9.6-kb *Eco*RI fragment of pMUS283 that carries part of *noeK*, *nodZ*, *noeL*, and part of *nolK*	This work
PMUS475	pMP92 containing a 2.7-kb *Apa*I fragment of pMUS424 that carries *noeL* and *nolK*	This work
PMUS519	Cosmid pLAFR1 containing a fragment of the HH103 symbiosis plasmid that carries the *noeL*::Tn*5*-B20 fusion	This work
pMP92	Broad-host-range cloning vector, Tc^r	Spaink et al. 1987

HCl at 80°C for 16 h. The methyl esters of fatty acids were identified by GC-MS. Location of the double bonds in the unsaturated fatty acyl residues was determined following preparation of dimethyl disulfide ethers of the methyl esters (Buser et al. 1983).

FAB-MS analysis.

Positive ion FAB mass spectra were obtained with MS1 of a JEOL JMS-SX/SX102A tandem mass spectrometer (JEOL, Tokyo) operated at 10 kV accelerating voltage. The FAB gun was operated at 6 kV accelerating voltage with an emission current of 10 mAmps and Xenon as the bombarding gas. Spectra were scanned at a speed of 30 s for the full mass range specified by the accelerating voltage used, and were recorded and averaged on an HP9000 data system (Hewlett Packard, Palo Alto, CA) running JEOL COMPLEMENT software. CID mass spectra were recorded with the same instrument, with helium as the collision gas in the third field free region collision cell, at a pressure sufficient to reduce the parent ion to one third of its original intensity. HPLC fractions were redissolved in 10 µl of dimethyl sulfoxide (DMSO) and 1-µl aliquots of sample solution were loaded into a matrix of monothioglycerol.

Plant assays.

Nodulation tests were carried out on *G. max* cv. Williams, *Albizia lebbeck* (L.) Benth., *A. lophantha* (Willd.) Huth, *Cajanus cajan* (L.) Millsp., *Desmodium canadense* (L.) DC., *Galega orientalis* Lam., *Lablab purpureus* (L.) Sweet, *Leucaena leucocephala* (Lam.) de Wit, *Macroptilium atropurpureum* (Moc. & Sessé ex DC.) Urb., *Neonotonia wightii* (J. Graham ex Arnott) J. Lackey, and *Vigna aconitifolia* (Jacq.) Maréchal, as described by Buendía-Clavería et al. (1989). Nitrogen fixation by nodules was assessed by acetylene reduction assays (Buendía-Clavería et al. 1986). Plant tops were dried at 70°C and weighed. Isolates from surface-sterilized nodules were tested on appropriately supplemented medium to determine whether they retained the antibiotic-resistance markers of the bacteria used to inoculate the plants. Competition studies were carried out on *G. max* cv. Williams as described by Romero et al. (1993).

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LITERATURE CITED

Allen, O. N., and Allen, E. K. 1981. The Leguminosae: A Sourcebook of Characteristics, Uses, and Nodulation. University of Wisconsin Press, Madison.

Appelbaum, E. R., Thompson, D. V., Idler, K., and Chartrain, N. 1988. *Rhizobium japonicum* USDA191 has two *nodD* genes that differ in primary structure and function. J. Bacteriol. 170:12-20.

Arnold, W., Rump, A., Klipp, W., Priefer, U. B., and Pühler, A. 1988. Nucleotide sequence of a 24,206-base-pair DNA fragment carrying the entire nitrogen fixation gene cluster of *Klebsiella pneumoniae*. J. Mol. Biol. 203:715-738.

Bec-Ferté, M.-P., Krishnan, H. B., Promé, D., Savagnac, A., Pueppke, S. G., and Promé, J.-C. 1994. Structures of nodulation factors from the nitrogen-fixing soybean symbiont *Rhizobium fredii*. Biochemistry 33: 11782-11788.

Bec-Ferté, M.-P., Krishnan, H. B., Savagnac, A., Pueppke, S. G., and Promé, J.-C. 1996. *Rhizobium fredii* synthesizes an array of lipooligosaccharides, including a novel compound with glucose inserted into the backbone of the molecule. FEBS Lett. 339:273-279.

Bellato, C., Krishnan, H. B., Cubo, T., Temprano, F., and Pueppke, S. G. 1997. The soybean cultivar specificity gene *nolX* is present, expressed in a *nodD*-dependent manner, and of symbiotic significance in cultivar-nonspecific strains of *Rhizobium* (*Sinorhizobium*) *fredii* strain USDA191. Microbiology 143:1381-1388.

Bergersen, F. J. 1961. The growth of *Rhizobium* in synthetic media. Aust. J. Biol. Sci. 14:349-360.

Beringer, J. E. 1974. R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. 84:188-198.

Blakeney, A. B., Harris, P. J., Henry, R. S., and Stone, B. A. 1984. A simple and rapid preparation of alditol acetates for monosaccharide analysis. Carbohydr. Res. 131:291-299.

Boyer, H. B., and Roulland-Dussoix, D. 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. 4:459-472.

Buendía-Clavería, A. M., Chamber, M., and Ruiz-Sainz, J. E. 1989. A comparative study of the physiological characteristics, plasmid content and symbiotic properties of different *Rhizobium fredii* strains. Syst. Appl. Microbiol. 12:210-215.

Buendía-Clavería, A. M., Rodriguez-Navarro, D. N., Santamaría, C., Ruiz-Saínz, J. E., and Temprano-Vera, F. 1994. Evaluation of the symbiotic properties of *Rhizobium fredii* in European soils. Syst. Appl. Bacteriol. 17:155-160.

Buendía-Clavería, A. M., Ruiz-Sainz, J. E., Cubo-Sanchez, T., and Perez Silva, J. 1986. Studies of symbiotic plasmids in *Rhizobium trifolii* and fast-growing bacteria that nodulate soybeans. J. Appl. Bacteriol. 61:1-9.

Buser, R. A., Arn, H., Guerin, P., and Rauscher, S. 1983. Determination of double-bond position in mono-unsaturated acetates by mass spectrometry of dimethyldisulphide. Anal. Chem. 55:818-822.

Chaplin, M. F. 1982. A rapid and sensitive method for the analysis of carbohydrate components in glycoproteins using gas-liquid chromatography. Anal. Biochem. 123:336-341.

Chen, W. X., Yan, G. H., and Li, J. L. 1988. Numerical taxonomic study of fast-growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. nov. Int. J. Syst. Bacteriol. 38:392-397.

Ciucanu, I., and Kerek, F. 1984. A simple method for the permethylation of carbohydrates. Carbohydr. Res. 131:209-217.

Dénarié, J., Debellé, F., and Promé, J.-C. 1996. *Rhizobium* lipochitooligosaccharide nodulation factors: Signaling molecules mediating recognition and morphogenesis. Annu. Rev. Biochem. 65:503-535.

Dowdle, S. F., and Bohlool, B. B. 1985. Predominance of fast-growing *Rhizobium japonicum* in a soybean field in the People's Republic of China. Appl. Environ. Microbiol. 50:1171-1176.

Fellay, R., Perret, X., Viprey, V., Broughton, W. J., and Brenner, S. 1995. Organization of host-inducible transcripts on the symbiotic plasmid of *Rhizobium* sp. NGR234. Mol. Microbiol. 16:657-667.

Freiberg, C., Fellay, R., Bairoch, A., Broughton, W. J., Rosenthal, A., and Perret, X. 1997. Molecular basis of symbiosis between *Rhizobium* and legumes. Nature 387:394-401.

Gabriel, O. 1973. Biological mechanisms involved in the formation of deoxy sugars: Enzymatic hydrogen mediation. A possible example of the evolutionary process of enzyme catalysis. Adv. Chem. 117:387-410.

Gerwig, G. J., Kamerling, J. P., and Vliegenthart, J. F. G. 1978. Determination of the D and L configurations of neutral monosaccharides by high-resolution capillary G. L. C. Carbohydr. Res. 62:349-357.

Gil-Serrano, A. M., Franco-Rodríguez, G., Tejero-Mateo, P., Thomas-Oates, J., Spaink, H. P., Ruiz-Sainz, J. E., Megías, M., and Lamrabet, Y. 1997. Structural determination of the lipo-chitin oligosaccharide nodulation signals produced by *Rhizobium fredii* HH103. Carbohydr. Res. 303:435-443.

Gil-Serrano, A. M., González-Jiménez, I., Tejero-Mateo, P., Bernabé, M., Jiménez-Barbero, J., Megías, M., and Romero-Vazquez, M. J. 1995. Structural analysis of the O-antigen of the lipopolysaccharide of *Rhizobium tropici* CIAT899. Carbohydr. Res. 275:285-294.

Goethals, K., Van Montagu, M., and Holsters, M. 1992. Conserved motifs in a divergent *nod* box of *Azorhizobium caulinodans* ORS571 re-

veal a common structure in promoters regulated by LysR-type proteins. Proc. Natl. Acad. Sci. USA 89:1646-1650.

Hernandez, B. S., and Focht, D. D. 1984. Invalidity of the concept of slow growth and alkali production in cowpea rhizobia. Appl. Environ. Microbiol. 48:206-210.

Heron, D. S., Érsek, T., Krishnan, H. B., and Pueppke, S. G. 1989. Nodulation mutants of *Rhizobium fredii* USDA257. Mol. Plant-Microbe Interact. 2:4-10.

Jabbouri, S., Fellay, R., Talmont, F., Kamalaprija, P., Burger, U., Relić, B., Promé, J.-C., and Broughton, W. J. 1995. Involvement of *nodS* in N-methylation and *nodU* in 6-O-carbamoylation of *Rhizobium* sp. NGR234 Nod factors. J. Biol. Chem. 270:22968-22973.

Jarvis, B. D. W., Downer, H. L., and Young, J. P. W. 1992. Phylogeny of fast-growing soybean-nodulating rhizobia supports synonymy of *Sinorhizobium* and *Rhizobium* and assignment to *Rhizobium fredii*. Int. J. Syst. Bacteriol. 42:93-96.

Keyser, H. H., Bohlool, B. B., Hu, T. S., and Weber, D. F. 1982. Fast-growing rhizobia isolated from root nodules of soybean. Science 215: 1631-1632.

Kondorosi, E., Pierre, M., Cren, M., Haumann, U., Buiré, M., Hoffmann, B., Schell, J., and Kondorosi, A. 1991. Identification of NolR, a negative transacting factor controlling the *nod* regulon in *Rhizobium* meliloti. J. Mol. Biol. 222:885-896.

Kovács, L. G., Balatti, P. A., Krishnan, H. B., and Pueppke, S. G. 1995. Transcriptional organization and expression of *nolXWBTUV,* a locus that regulates cultivar-specific nodulation of soybean by *Rhizobium fredii* USDA257. Mol. Microbiol. 17:923-933.

Krishnan, H. B., and Pueppke, S. G. 1991. Sequence and analysis of the *nodABC* region of *Rhizobium fredii* USDA257, a nitrogen-fixing symbiont of soybean and other legumes. Mol. Plant-Microbe Interact. 4:512-520.

Krishnan, H. B., and Pueppke, S. G. 1994a. Cultivar-specificity genes of the nitrogen-fixing soybean symbiont, *Rhizobium fredii* USDA257, also regulate nodulation of *Erythrina* spp. Am. J. Bot. 81:38-45.

Krishnan, H. B., and Pueppke, S. G. 1994b. Host range, RFLP, and antigenic relationships between *Rhizobium fredii* strains and *Rhizobium* sp. NGR234. Plant Soil 161:21-29.

Krishnan, H. B., Kuo, C.-L., and Pueppke, S. G. 1995. Elaboration of flavonoid-induced proteins by the nitrogen-fixing soybean symbiont *Rhizobium fredii* is regulated by both *nodD1* and *nodD2,* and is dependent on the cultivar-specificity locus, *nolXWBTUV*. Microbiology 141:2245-2251.

Krishnan, H. B., Lewin, A., Fellay, R., Broughton, W. J., and Pueppke, S. G. 1992. Differential expression of *nodS* accounts for the varied abilities of *Rhizobium fredii* USDA257 and *Rhizobium* sp. NGR234 to nodulate *Leucaena* spp. Mol. Microbiol. 6:3321-3330.

López-Lara, I. M., Blok-Tip, L., Quinto, C., Garcia, M. L., Stacey, G., Bloemberg, G. V., Lamers, G. E. M., Lugtenberg, B. J. J., Thomas-Oates, J. E., and Spaink, H. P. 1996. NodZ of *Bradyrhizobium* extends the nodulation host range of *Rhizobium* by adding a fucosyl residue to nodulation signals. Mol. Microbiol. 21:397-408.

Lorquin, J., Lortet, G., Ferro, M., Méar, N., Dreyfus, B., Promé, J.-C., and Boivin, C. 1997a. Nod factors from *Sinorhizobium saheli* and *S. teranga* bv. *sesbaniae* are both arabinosylated and fucosylated, a structural feature specific to *Sesbania rostrata* symbionts. Mol. Plant-Microbe Interact. 10:879-890.

Lorquin, J., Lortet, G., Ferro, M., Méar, N., Promé, J.-C., and Boivin, C. 1997b. *Sinorhizobium teranga* bv. *acaciae* ORS1073 and *Rhizobium* sp. strain ORS1001, two distantly related *Acacia*-nodulating strains, produce similar Nod factors that are O carbamoylated, N methylated, and mainly sulfated. J. Bacteriol. 179:3079-3083.

Maniatis, T. A., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Meinhardt, L. W., Krishnan, H. B., Balatti, P. A., and Pueppke, S. G. 1993. Molecular cloning and characterization of a sym plasmid locus that regulates cultivar-specific nodulation of soybean by *Rhizobium fredii* USDA257. Mol. Microbiol. 9:17-29.

Mergaert, P., D'Haeze, W., Fernández-López, M., Geelen, D., Goethals, K., Promé, J.-C., Van Montagu, M., and Holsters, M. 1996. Fucosylation and arabinosylation of Nod factors in *Azorhizobium caulinodans:* Involvement of *nolK, nodZ* as well as *noeC* and/or downstream genes. Mol. Microbiol. 21:409-419.

Price, N. P. J., Relić, B., Talmont, F., Lewin, A., Promé, D., Pueppke, S. G., Maillet, F., Dénarié, J., Promé, J.-C., and Broughton, W. J. 1992. Broad-host-range *Rhizobium* species strain NGR234 secretes a family of carbamoylated, and fucosylated, nodulation signals that are O-acetylated or sulphated. Mol. Microbiol. 6:3575-3584.

Quesada-Vincens, D., Fellay, R., Nasim, T., Viprey, V., Burger, U., Promé, J.-C., Broughton, W. J., and Jabbouri, S. 1997. *Rhizobium* sp. strain NGR234 NodZ protein is a fucosyltransferase. J. Bacteriol. 179:5087-5093.

Quinto, C., Wijfjes, A. H. M., Bloemberg, G. V., Blok-Tip, L., López-Lara, I., Lugtenberg, B. J. J., Thomas-Oates, J. E., and Spaink, H. P. 1997. Bacterial nodulation protein NodZ is a chitin oligosaccharide fucosyltransferase which can also recognize related substrates of animal origin. Proc. Natl. Acad. Sci. USA 94:4336-4341.

Roche, P., Lerouge, P., Ponthus, C., and Promé, J.-C. 1991. Structural determination of bacterial nodulation factors involved in the *Rhizobium* meliloti-alfalfa symbiosis. J. Biol. Chem. 266:10933-10940.

Rodríguez-Navarro, D. N., Ruiz-Sainz, J. E., Buendía-Clavería, A. M., Santamaría, C., Balatti, P. A., Krishnan, H. B., and Pueppke, S. G. 1996. Characterization of fast-growing rhizobia from nodulated soybean [*Glycine max* (L.) Merr.] in Vietnam. Syst. Appl. Microbiol. 19: 240-248.

Romero, F., Buendía-Clavería, A. M., and Ruiz-Sainz, J. E. 1993. Broad host-range effective mutants of *Rhizobium fredii* strains. J. Appl. Bacteriol. 74:610-619.

Schultze, M., Quiclet-Sire, B., Kondorosi, É., Virelizier, H., Glushka, J. N., Endre, G., Géro, S. D., and Kondorosi, A. 1992. *Rhizobium meliloti* produces a family of sulfated lipo-oligosaccharides exhibiting different degrees of plant host specificity. Proc. Natl. Acad. Sci. USA 89:192-196.

Shen, B., and Davis, L. C. 1992. Nodulation and nodulin gene expression in an interspecies hybrid between *Glycine max* and *Glycine tomentella*. Aust. J. Plant Physiol. 19:693-707.

Simon, R. 1984. High frequency mobilization of Gram negative bacterial replicons by the in vivo constructed Tn*5*-Mob transposon. Mol. Gen. Genet. 196:413-420.

Simon, R., Priefer, U., and Pühler, A. 1983. A broad host range mobilization system for in vivo genetic engineering: Transposon mutagenesis in Gram negative bacteria. Bio/Technology 1:784-791.

Simon, R., Quandt, J., and Klipp, W. 1989. New derivatives of transposon Tn*5* suitable for mobilization of replicons, generation of operon fusions and induction of genes in Gram-negative bacteria. Gene 80: 161-169.

Spaink, H. P., Okker, R. J. H., Wijffelman, C. A., Pees, E., and Lugtenberg, B. J. J. 1987. Promoters in the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1JI. Plant Mol. Biol. 9:27-39.

Stacey, G., Luka, S., Sanjuan, J., Banfalvi, Z., Nieuwkoop, A. J., Chun, J. Y., Forsberg, L. S., and Carlson, R. 1994. *nodZ,* a unique host-specific nodulation gene, is involvedin the fucosylation of the lipo-oligosaccharide nodulation signal of *Bradyrhizobium japonicum*. J. Bacteriol. 176:620-633.

Vinardell, J. M. 1997. Estudios fisiológicos y genéticos del gen regulador *nodD* de *Rhizozobium fredii* HH103. Ph.D. dissert. University of Seville, Spain.

Young, C. C., Chang, J. Y., and Chao, C. C. 1988. Physiological and symbiotic characteristics of *Rhizobium fredii* isolated from subtropical-tropical soils. Biol. Fertil. Soils 5:350-354.